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Producing Glutathione, Carotene and Proline in Chlorella sorokinana on the Effect of Salt Stress and Different Growth Phases

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Abstract. *This study explained the effect of Nacl added to the culturing medium Bg11 during the growth of Chlorella sorokinana. It showed that there was a direct relationship between the increase in the concentration of Nacl and both glutathione and proline; the maximum concentrations recorded were 75.234 mg.l-1 and 27.218 mg L-1, correspondingly, at the level of 300 ppm. Conversely, The maximum level of carotene identified was 1.6 mg.l-1 when using a dosage of 100 ppm. The findings from the comparison of growth phase dates indicated that the peak values for glutathione, proline, and carotene were 71.238, 22.359, and 1.625 mg.l-1 respectively during the second growth phase. The results of the interaction analysis revealed that the highest levels of both glutathione and proline were 78.873 and 30.166 mg.l-1 respectively when the concentration was 300 ppm in the second growth phase. Regarding carotene, the maximum value reached was 2.5 mg.l-1 at a concentration of 100 ppm during the second growth phase. From the present study, we conclude that the Nacl concentration of 300 ppm was the most suitable for obtaining the highest production of glutathione and proline, whereas the optimal Nacl concentration for obtaining the highest production of carotene was at 100 ppm. Finally, growth phases in the second phase of growth was the best age phase for getting the highest production of glutathione, proline and carotene.*

Keywords: *Carotene, Chlorella sorokinana, Glutathione, growth phases, NaCl, Proline.*

INTRODUCTION

Microalgae are aquatic organisms that contain photosynthetic pigments and have microscopic, unicellular shapes. Their biomass is a potential the origin of biologically active compounds that possess significance in the fields of medicine and pharmaceuticals [1],[2]. Chlorella is considered one of the most widespread genera of microalgae. It involves approximately one one hundred species of unicellular green algae are documented in the algae database. Chlorella strains have the ability to produce secondary metabolites, which are widely used in many industrial fields [3]. Ch. sorokinana is a microalgae that is characterized by its easy and Rapid growth and ability to generate secondary metabolites. such as steroids, flavonoids, and saponins, as well as lipids, carbohydrates, and amino acids, especially when using ethanolic extracts [4].

Glutathione is regarded as one of the main antioxidants that maintains the balance of oxidation and reduction states in neurons. It has an important role in upholding the physiological processes of every cell within the organism.. This plays a crucial role in the defense system, knowing that depleting this compound in the brain leads to neurodegenerative diseases such as Alzheimer [5]. In most diseases, which are caused by the oxidative stress as it is believed, the concentration of glutathione decreases largely and this makes cells more susceptible to damage. Accordingly, there is increasing interest in identifying the best ways to increase glutathione for preventing and treating diseases [6].

The exposure to stress besides its role in accumulating secondary metabolites leads to accumulate organic and amino acids. It was found out that the accumulation of amino acid of proline occurred in response to many abiotic stresses, and that the accumulation of this acid is usually resulted from the hydrolysis or destruction of proteins. It was discovered that it has an important role in making plants tolerate stress by maintaining osmotic balance; this in turn has encouraged searching for different methods to manipulate the biosynthesis of proline in order to protect algae from various stresses [7]. Proline is considered an amino acid having multiple functions and its accumulation is closely related to many cellular processes like osmotic stress, changes in redox balance, reduction, and defenses against pathogens.

Also, proline biosynthesis is associated with photosynthesis and respiration. Many studies have shown close relationships between environmental effects and proline synthesis [8]. Photosynthetic pigments are compounds with biological activities. They have been classified into several types including carotenoids which can be used as antioxidants, anti-inflammatory agents, and immune system stimulants. Carotenoids are produced by certain species as main products and in some species as secondary products based on metabolic pathways. Due to their bioactive capacity, the demand for these pigments has increased as nutritional supplements and pigments, besides their role in pharmaceutical and cosmetic applications [9].

Microalgae have carotenoids which are mainly used to improve the efficiency of light energy and protect chlorophyll pigments from photooxidation. Some of them such as α-carotene, β-carotene, and β-cryptoxanthin are carotenoids that are initiators of vitamin A. Carotenoids are considered as antioxidants that protect cells and tissues from the harmful effects of free radicals [10],[11]. Carotenoids are regarded as a crucial component of the human diet; it plays a major role in human nutrition and health [12]. In addition, its properties in eliminating free radicals make it suitable as a source of natural antioxidants. Many studies have focused on selecting promising strains of algae that produce carotenoids and secondary metabolites and work on extracting and purifying them [13].

MATERALS AND METHODS

The investigation was conducted in aseptic conditions within the Plant Tissue Culture facility situated in the Department of Biology at the College of Education for Pure Sciences at the University of Diyala. A culture of Chlorella sorokinana (cultivated at room temperature) was maintained at 2±25°C with an 8/16 hour light-dark cycle exposing them to a light intensity of 3000 lux. To ensure constant movement, the samples were positioned on a shaker.

a. Preparation of the Culturing Media

Chlorella sorokinana was grown on Bg-11 culturing medium which was prepared by taking 1.6g of culturing medium and dissolving it in 1L and autoclaving (118°C and the pressure of 121bar) for 15 min.

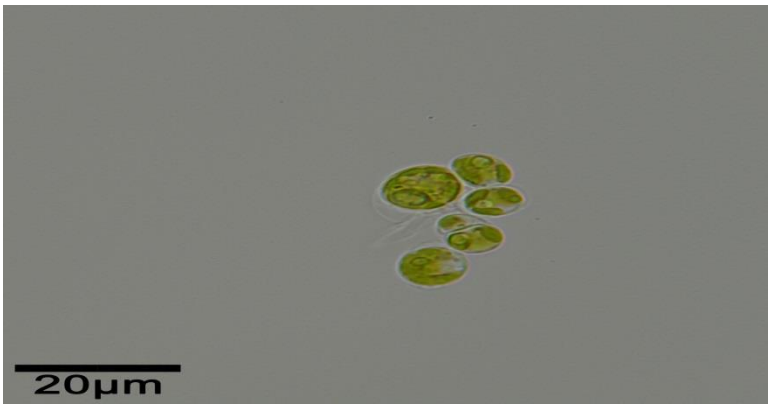

Figure 1. The Shape of *Chlorella sorokinana* on Electronic Microscope.

b. Preparation of the Isolate

Only 900ml of sterilised culturing medium was taken and the volume which was 1L was completed with the algal isolate.

c. Preparation of Sodium Chloride

The required weights of NaCl (0, 0.1, 0.2 and 0.3 ppm) were prepared and added to the culturing media containing the algal isolate.

d. Harvesting the Cells

Chlorella sorokinana cells were harvested three times during the experiment period throughout the growth phases of each group of the experimental unit. The first harvest (the first phase) was after 7 days, the second harvest (the second phase) was after 14 days, and the third harvest (the third phase) was after 21 days [14].

e. Diagnosis and Quantitative Estimation of Glutathione

The mobile phase was an isocratic flow of a 50/50 (v/v) mixture of water (pH 7.0) and acetonitrile flow rate at 1.0 mL/min , column was C18 – ODS (25 cm * 4.6 mm) and the detector florescence (Ex = 445 nm , Em = 465 nm) [15].

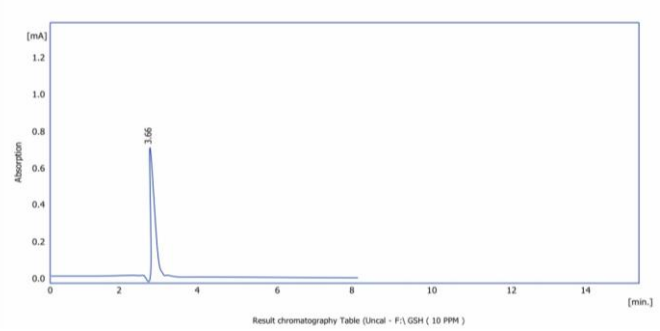

Figure 2. Standard Curve of Glutathione

f. Diagnosis and Quantitative Estimation of Proline

Approximately 5 ml of specimens were extracted and combined with 1 ml of a 1 (molary) hydrochloric acid solution serving as the hydrolysis agent. Subsequently, the tube was shielded and positioned within the aluminum thermos block at a temperature of 40°C±2°C for a duration of 5 hours to facilitate the hydrolysis process. Following this, utilizing a pipette, a 100 µl portion of the hydrolyzed substance was introduced into a vial situated in an evaporation setup to eliminate moisture using nitrogen gas. The desiccated amino acid remnants were solubilized in a 100 µl volume of acetonitrile and then derivatized with a 100 µl amount of ortho phthalaldehyde. The hermetically sealed vial was subjected to ultrasound for a period of 1 minute. Subsequently, the vial was positioned within the thermos block at a temperature of 50°C±2°C for 30 minutes to finalize the process of derivatization was carried out, followed by the transfer of the vial to the sample stand of the gas chromatograph. A total of 100 injections, each consisting of 100 µl per sample, were then performed [16].

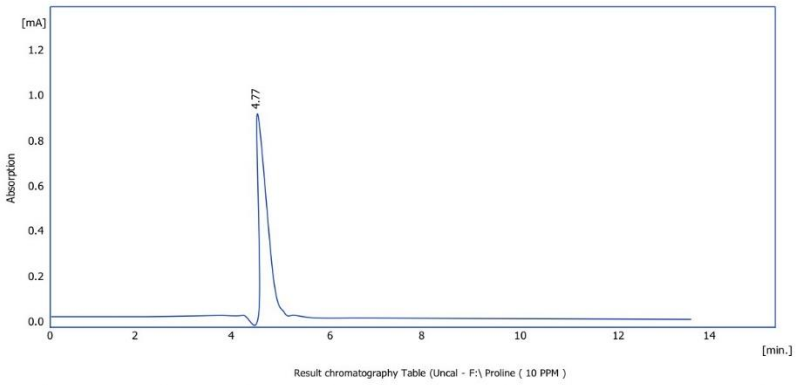

Figure 3. Standard Curve of proline

g. Carotene estimation

Carotene was estimated by using (2.5)cm3 of ice-cold acetone at the concentration of 80% with (7.5) cm3 of algal isolate. It was placed in a centrifuge at 3500 rpm for (10) minutes. Only (1) cm3 of the filtrate was taken to measure the absorbance in the spectrophotometer. The measurement was the wavelength of 480 and the estimation was done according to the following equation:

Carotene = 200 × absorbance at 480 nm [17].

h. Experiment Design

The experiment was designed by using CRD with three replicates for each treatment and the results were analyzed via using SPSS program at the significance level of 0.05.

RESULTS

The results of Table (1) showed that the highest value of producing glutathione was at the NaCl concentration of 300 ppm; it was 75.284 mg.l-1, while the lowest value was in the control treatment and it was 62.220 mg.l-1. Concerning the difference in the growth phases, the results showed that the highest value was 71.238 mg.L-1 in the second phase of growth, whereas the lowest value was 66.266 mg.l-1 in the first phase.

Table 1. Effect of different concentrations of NaCl, the difference of growth phases and their interaction on the glutathione concentration in *Chlorella sorokinana*

Phases	Concentration Nacl (Ppm) Gsh				**Effect Of Phases**
	Control	**100**	**200**	**300**	
Phases 1	60.730 l	65.870 i	67.873 g	71.220 d	**66.266 c**
Phases 2	63.366 j	69.163 f	73.556 c	78.873 a	**71.238 a**
Phases 3	62.463 k	66.690 h	70.220 e	75.586 b	**68.765 b**
Effect of NaCl	62.220 d	67.241 b	70.241 b	75.284 a	

The findings presented in Table (2) revealed an escalation in proline levels, indicating a direct correlation with the rise in NaCl concentration. At 300 ppm, the peak value recorded was 27.218 mg.l^{-1}, whereas it plummeted to 13.060 mg.l^{-1}1 in the control group. Analysis of cell harvesting dates indicated a maximal value of 22.359 mg.l^{-1} during the second phase, contrasting with a minimal value of 17.923 mg.l^{-1} in the initial growth phase.

Table 2. Effect of different concentrations of NaCl, the difference of growth phases and their interaction on the proline concentration in *Chlorella sorokinana*

Phases	Concentration Nacl (Ppm) Proline				**Effect Of Phases**
	Control	**100**	**200**	**300**	
Phases 1	12.383 L	16.163 I	19.513 G	23.633 D	**17.923 C**
Phases 2	13.740 J	20.113 F	25.416 C	30.166 A	**22.359 A**

Phases 3	13.056 K	17.880 H	22.550 E	27.856 B	**20.335 B**
Effect Of Nacl	13.060 D	18.052 C	22.493 B	27.218 A	

The results of Table (3) showed that the effect of NaCl concentration 100 ppm was the best in obtaining the highest value of carotene, which was 1.6 $mg.l^{-1}$. Analysis of cell harvesting dates indicated a maximal value of 1.625 $mg.l^{-1}$ during the second phase, contrasting with a minimal value of 0.475 $mg.l^{-1}$ in the third growth phase.

Table 3. Effect of different concentrations of NaCl, the difference of growth phases and their interaction on the caroten concentration in *Chlorella sorokinana*

Phases	Concentration Nacl (Ppm) Caroten				**Effect Of Phases**
	Control	**100**	**200**	**300**	
Phases 1	0.431 H	1.8 C	0.96 De	0.70 G	0.983 B
Phases 2	2.06 B	2.5 A	1.06 D	0.86 F	1.625 A
Phases 3	0.433 H	0.767 Fg	0.467 H	0.23 I	0.475 C
Effect Of Nacl	0.974 B	1.6 A	0.83 C	0.60 D	

a. Discussion

Salinity causes osmotic stress which contributed to involve many adaptations such as osmotic adjustment, hormonal regulation, and the antioxidant defense system which consists of enzymatic and non-enzymatic components such as glutathione working to reduce ROS that increases with the increase of the environmental stresses [18]. Several studies indicated that oxidative stress enhances the covalent modification of proteins by glutathione and this modification is called S-glutathionylation [19]. sThe increase in glutathione after 14 days of growth might be attributed to abiotic stress which stimulated the immune system for maintaining the completion of the cells life cycle. This leads to the consumption of materials resulting from photosynthesis which was followed by an increase in the production of antioxidant compounds to prevent free radicals [21]. The production of secondary metabolites is affected by the difference in growth phases as well as the different environmental conditions.

The build-up of proline due to stress conditions primarily stems from its biosynthesis and reduction in breakdown. This accumulation of proline is crucial for plants to adapt to stress by aiding in the maintenance of cell membranes and regulation of oxidation-reduction processes.. It can act as a signal molecule to modify the mitochondria functions [22]. Also, proline, an amino acid, is recognized as an osmolyte that is widely distributed and remarkably efficient in its function. Its primary role involves safeguarding cellular structures against the detrimental effects of osmotic stress. When cells are subjected to elevated levels of salt within their surrounding milieu, a phenomenon occurs where water molecules migrate from the cytosol to the extracellular space. Consequently, cellular dehydration and subsequent protein degradation are observed. Proline emerges as a pivotal agent in maintaining cellular hydration, thereby counteracting the loss of water induced by osmotic pressure[23]. That accumulation of proline is a defense means that algae used when exposing to stresses [24]. As for the difference in the growth phases, the outcomes could potentially be ascribed to the observation that the concentration of amino acids exhibited a gradual decline as the algae proliferated, reaching their peak production levels upon reaching the stationary phase of growth [25]. Proline acid is one of the multifunctional amino acids as it regulates many vital processes [26].

These results explained that NaCl has an effect on the genes stimulating carotenoids; knowing that the gene expression stimulating carotenoids varies according to the stress [27]. When studying the effect of salt stress on the microalga *Chlamydomonas reinhardtii*, stated that this stress increased the carotene content in the alga when using the salt concentration of 0.02 M [28]. Concerning the difference in the growth phases, the results indicated that the highest value of the carotene concentration mean was 1.625 $mg.L^{-1}$ in the second phase of growth, while it decreased to reach its lowest value 0.475 $mg.L^{-1}$ in the third phase. This result might be explained by the difference in the functional properties of the cell membrane that were affected by the difference in the growth phases besides the gradual depletion of nutrients in the culturing medium which in turn negatively affected the concentration of carotenoids [29]. In regards to the outcomes of the interplay among various levels of NaCl concentrations during distinct growth stages, the peak amount of carotenoids recorded was 2.5 mg L-1 observed at a concentration of 100 ppm during the second growth phase.. This was consistent with [30] in that the concentration of carotenoids increased when NaCl was added to the culturing medium of *Coelastrella* sp. and harvesting the cells in the stationary phase.

CONCLUSIONS

It is obvious from the above results that the concentration of 300 ppm of NaCl is the best in stimulating the algal cells to produce glutathione and proline. Regarding to the carotene pigment, it was found that the concentration of 100 ppm contributed to stimulate the production of these pigments. On the other hand, it was found that 14 days of algae growth is the most appropriate time for harvesting algal cells to obtain the highest productivity in each of glutathione, proline and carotene.

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***Conflict of Interest Statement**:The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.*